Exhibit A

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Methods and Protocols

Edited by Paul T. Sharpe and Ivor Mason

MOLECULAR EMBRYOLOGY

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described in this chapter. The approach enables stable expression of cloned gene products in *Xenopus* embryos, allowing a broader range of feasible experimentation than that previously possible by transient expression methods.

Unlike plasmid injection, transgenesis allows stable, temporally and spatially controlled expression of gene products in desired cells of the *Xenopus* embryo. We have used transgenesis to express genes of interest ectopically, to direct expression of modified gene products which dominantly interfere with the function of their endogenous, wild-type counterparts, and to analyze the spatial regulation of promoters in the embryo *(1)*. We have been able to obtain large numbers of transgenic embryos readily for these purposes and to interpret reliably the effect of transgene expression without the cell-to-cell variability of expression within an embryo, which plagues many studies using plasmid-injected embryos.

1.3. Overview of Transgenesis Procedure

In the transgenesis approach described here, DNA is integrated into isolated sperm nuclei in vitro, followed by transplantation of the nuclei into unfertilized eggs, thus generating transgenic embryos. Nuclear transplantation of transfected cultured cells was previously used by one of us to produce transgenic *Xenopus* embryos, which expressed promoter-reporter plasmids nonmosaically *(2)*. However, the cultured cells used as nuclear donors for these transplantations were aneuploid and rarely promoted development of the pseudo-triploid embryos to tadpole stages. To overcome these problems, we now use sperm nuclei to generate transgenic embryos. These nuclei offer many advantages over cultured cell lines. First, since sperm nuclei are haploid, there is no need to destroy the egg nucleus before transplantation to generate a normal diploid embryo. Second, sperm nuclei have been used for many years to investigate the processes of chromosome decondensation, nuclear assembly, and cell-cycle progression *(3–5)*. These studies have provided us with valuable information regarding the manipulation of sperm nuclei in vitro. Indeed we have discovered that we can introduce DNA into sperm nuclei swelled and decondensed in cell-free egg extracts using restriction enzyme-mediated integration (REMI) *(6,7)*. When these nuclei are transplanted into unfertilized eggs, we obtain large numbers of normal diploid tadpoles, which develop to advanced stages and express inserted genes at high frequency.

The protocol for *Xenopus* transgenesis described here involves the following steps:

1. Sperm nuclei are incubated with linearized plasmid DNA.
2. After a short incubation, a high-speed interphase egg extract and a small amount of the restriction enzyme used for plasmid linearization are added to the sperm nuclei/plasmid mixture. The extract partially decondenses sperm chromatin, but does not promote replication.

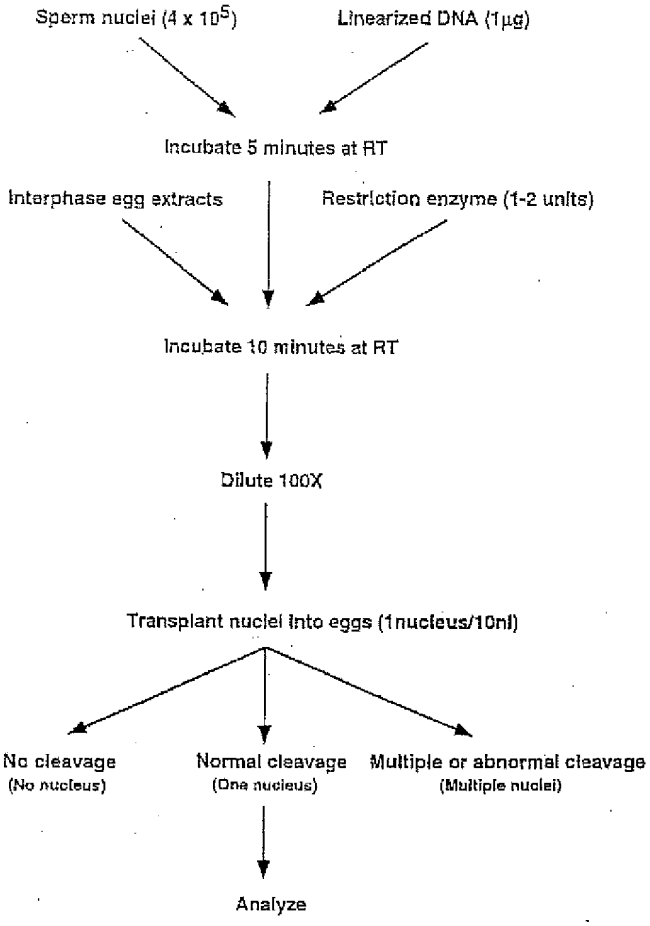

Fig. 1. Overview of transgenesis procedure. Sperm nuclei are incubated with linear DNA for a brief period of time. Interphase egg extracts and a restriction enzyme are then added. The egg extracts partially decondense the chromosomes, and the restriction enzyme very lightly cleaves them. These events facilitate the eventual integration of the linear DNA into the chromosomes. After incubation of nuclei in a mixture of extract, restriction enzyme, and plasmid DNA, the nuclei are diluted, and approximately one nucleus is transplanted per egg. Each activated egg requires a nucleus (or at least the centriole introduced with a nucleus) to divide; therefore, only eggs receiving a nucleus develop into embryos. Eggs that receive more than one nucleus (polyspermic eggs) divide abnormally into multiple cells at the first cleavage division. Embryos developing from monospermic eggs cleave normally during early divisions; only these embryos are isolated and analyzed. Generally, between 20 and 50% of these embryos will be transgenic.

1.5. Analysis of DNA Integration in Transgenic Embryos

We have analyzed genomic DNA from transplantation-derived tadpoles to determine whether early integration of introduced plasmids into sperm or egg

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allowed us to very simply produce normal embryos from oocytes handled in vitro. A similar approach has also been developed to overcome male infertility in humans *(17–20)*.

2. Materials

2.1. Sperm Nuclei Preparation

1. 1X Nuclear Preparation Butter (NPB): 250 m*M* sucrose (1.5 *M* stock; filter-sterilize and store aliquots at –20°C), 15 m*M* HEPES (1 *M* stock; titrate with KOH so that pH 7.7 is at 15 m*M*, filter-sterilize, and store aliquots at –20°C) (*see* **Note 4**), 0.5 m*M* spermidine trihydrochloride (Sigma S-2501; 10 m*M* stock; filter-sterilize and store aliquots at –20°C), 0.2 m*M* spermine tetrahydrochloride (Sigma S-1141; 10 m*M* stock; filter-sterilize and store aliquots at –20°C), 1 m*M* dithiothreitol (Sigma D-0632; 100 m*M* stock; filter-sterilize and store aliquots at –20°C) (*see* **Notes 4** and **5**).

For steps requiring protease inhibitors, add: 10 µg/mL leupeptin (Boeringer Mannheim 1 017 101; 10 mg/mL stock in DMSO, store aliquots at –20°C), 0.3 m*M* phenylmethylsulfonyl fluoride (PMSF) (Boeringer Mannheim 837 091; 0.3 *M* stock in EtOH, stored at –20°C).

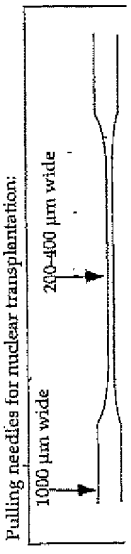
2. 1X Marc's Modified Ringer (MMR): 100 m*M* NaCl, 2 m*M* KCl, 1 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, 5 m*M* HEPES, pH 7.5. Prepare a 10X stock, and adjust pH with NaOH to 7.5. Sterilize 10X and 1X solutions by autoclaving.
3. Lysolecithin: 100 µL of 10 mg/mL L-α-lysophosphatidylcholine (Sigma Type I, L-4129); dissolve at room temperature just before use. Store solid stock at –20°C.
4. Bovine serum albumin (BSA) (store at –20°C in 1 mL aliquots): 10% (w/v) BSA (fraction V, Sigma A-7906; prepare stock in water, titrate to pH 7.6 with KOH).
5. Sperm dilution buffer (store at -20°C in 0.5-mL aliquots): 250 m*M* sucrose, 75 m*M* KCl, 1 m*M* EDTA (0.5 *M* stock, pH8), 0.5 m*M* spermidine trihydrochloride (Sigma S-2501; 10 m*M* stock; filter-sterilize and store aliquots at –20°C), 0.2 m*M* spermine tetrahydrochloride (Sigma S-1141; 10 m*M* stock; filter-sterilize and store aliquots at –20°C), 1 m*M* dithiothreitol (Sigma D-0632; 100 m*M* stock; filter-sterilize and store aliquots at –20°C).
6. Hoechst No. 33342 (Sigma B-2261): 10 mg/mL stock in dH_2O; store in a light-tight vessel at –20°C.

2.2. High-Speed Egg Extract Preparation

1. 20X Extract buffer (XB) salt stock: 2 *M* KCl, 20 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, filter-sterilize and store at 4°C.
2. Extract buffer (XB): 1X XB salts (100 m*M* KCl, 0.1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$; from 20X XB salts stock solution), 50 m*M* sucrose (1.5 *M* stock; filter-sterilize and store in aliquots at –20°C), 10m*M* HEPES (1 *M* stock, titrated with KOH such that pH is 7.7 when diluted to 10 m*M*; should require about 5.5 mL of 10 *N* KOH for 100 mL; filter-sterilize, and store in aliquots at –20°C) (*see* **Note 4**). Prepare about 100 mL.

We have used enzymes purchased from Boeringer Mannheim or New England Biolabs for transplantation reactions. Some calibration may be required to determine the optimal amount of enzyme to add to each reaction, since additions of 0.5 µL of undiluted enzyme to reactions can adversely affect the development of nuclear transplant embryos. We generally test several dilutions of enzyme (1:2.5, 1:5; 1:10) to identify a dose that has no apparent deleterious effects on transplant embryo development when compared with embryos produced with no enzyme addition.

9. Agarose-coated injection dishes: 2.5% agarose in dH_2O is poured into 35-mm or 60-mm Petri dishes. Before the agarose solidifies, a well template (a rectangular square of Dow-Corning Sylgard 184 elastomer) is laid onto it. After the agarose has solidified and the Sylgard templates have been removed, 1X MMR is poured into each dish to prevent dehydration. The dishes are then wrapped in parafilm and stored at 4°C (weeks to months) until use.
10. Transplantation needles: 30-µL Drummond micropipets (Fisher, cat. #: 21-170J) are pulled to produce large needles with long, gently sloping tips (**Fig. 2**). A micropipet (1 mm wide; 8 cm long) is first heated in a Bunsen burner flame and drawn by hand to make the bore of the needle (200–400 µm wide). This drawn pipet should be 10–15 cm in length and should remain fairly straight when held by one end. To produce a gently sloping needle tip, this pipet is drawn again. We use a gravity-driven needle puller for this: the upper end of the needle is fixed in a brace, the center of the needle bore of the drawn pipet is placed within a small heating coil, and a weight is attached to the lower end of the needle. The gravity driven pullers we have used are home-built and about 10–20 yr old, but similar vertical pullers are commercially available from Narishige (i.e., Model PB-7). The second pull can also be performed with a horizontal needle puller available from Sutter Instrument Co. (Model P-87; Flaming/Brown micropipet puller) using settings like those used to make other injection needles. In limited trials of the Sutter puller using a standard setting, we have found that the needles produced had a steeper slope near the tip and were slightly more difficult to use than those drawn with our vertical puller; however, settings can probably be adjusted on this and other commercially available pullers to produce long, gently sloping tips that will work well for transplantation. Needles are clipped with a forceps to produce a beveled tip of 60–75 µm diameter (*see* inset in **Fig. 2**), using the ocular micrometer of a dissecting microscope for measurement.
11. Transplantation apparatus: We have found most commercial injection apparatuses commonly used for RNA and DNA injections unsuitable for nuclear transplantation. This is largely due to the difference in needle tip size. Flow through the 5–10 µm needle tips used for fluid injections can be controlled at fairly high pressures. However, with standard air-injection systems, we have been unable to obtain the extremely low positive pressure, and gentle, controlled flow required to deliver an intact nucleus in a small volume (10–15 nL) through the 50–70 µm tips of nuclear transplantation needles. Oil-filled injection systems (Drummond) are likely to work, since they are based on a positive displacement mechanism that should not be affected by the tip size of the needle. At this writing, though,



we have not tried one of these injection apparatuses for nuclear transplantations. Instead, we will describe how to make a home-made air injection apparatus that works extremely well for nuclear transplantions on a large scale and that costs very little (approx $200).

The transplantation apparatus that has given us the most success is shown in **Fig. 2**. A line connects the house vacuum outlet to a three way valve. Another line connects the house air outlet to a T-connector that splits the air flow into an exhaust line and another line connecting to the three way valve. Finally, another line connects the three way valve to the needle. For fine control of the positive pressure into the needle a screw clamp is placed on the exhaust line. Screwing down on this clamp increases the positive pressure into the system, while opening the clamp decreases the positive pressure. Negative pressure is established by opening slightly the valve (on the three way valve) connected to the house vacuum line. A more rough adjustment of positive pressure also can be obtained by opening or closing the valve (on the three way valve) connected to the house air line. By using a combination of these adjustments, we are able to obtain a very slow, controllable flow through a 50–70-µm needle. As flow is continuous, transplantations can usually be done more rapidly than injections of RNA or DNA, since it is only necessary to move from egg to egg to deliver nuclei. Parts needed to build the transplantation apparatus shown in **Fig. 2** are listed in **Table 1**.

Alternatively, a transplantation apparatus like the one shown in **Fig. 3** can be constructed. For this apparatus, a large, air-filled Hamilton Syringe (30 cc Multifit Interchangeable syringe with Luer-Lok tip; Fisher) is connected to a length of Tygon tubing. A metal plunger removed from a Syringe Microburet (Model # SB2; Micro-metric Instrument Co., Cleveland, OH) is used to control injection of the nuclei. We have found this apparatus usable although it is not controlled as easily as the one shown in **Fig. 2**.

3. Methods

3.1. Transgenesis Method (see Note 3)

3.1.1. Sperm Nuclei Preparation

We have generally followed the standard protocol of Murray *(4)*, but have omitted the protease inhibitors leupeptin and phenylmethylsulfonyl fluoride from many steps to avoid transfer into the final mixture, which is diluted for egg injections.

1. Dissect and isolate the testes from a male:
   a. Anesthetize a male in a bucket containing a liter of 0.1% Tricaine (MS222, aminobenzoic acid ethyl ester, Sigma A-5040) and 0.1% sodium bicarbonate for at least 20 min (immersion of the animal in ice water for 20 min may also be used), and pith it.
   b. Cut through the ventral body wall and musculature, and lift the yellow fat bodies to isolate the two testes, which are attached to the base of the fat bodies, one on each side of the midline.

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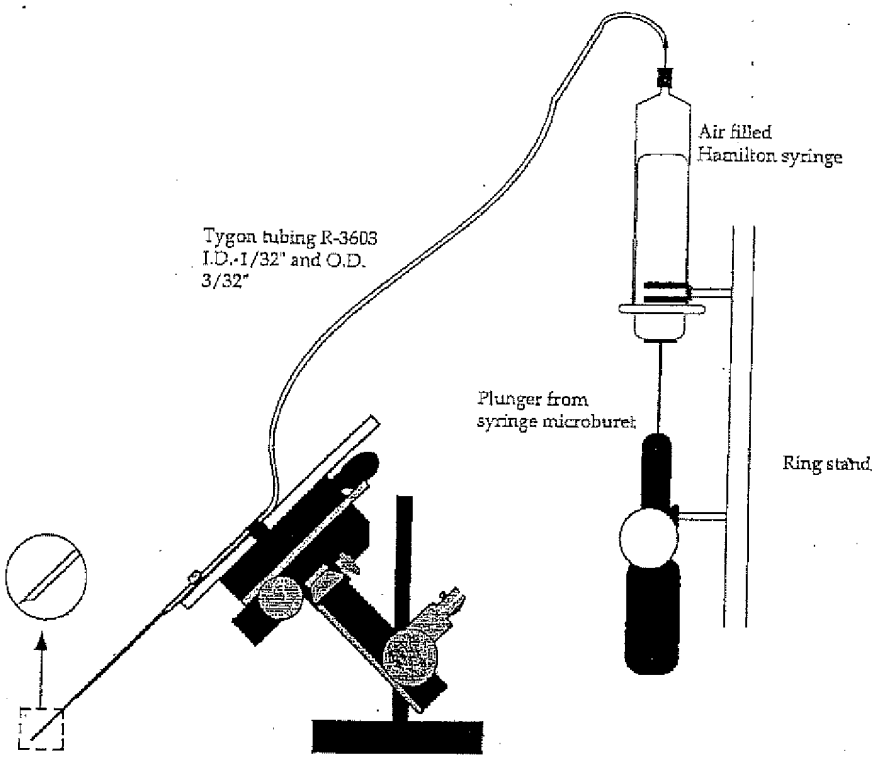

Fig. 3. Diagram of alternative injection apparatus. A line connects the needle to a 30-mL Hamilton syringe that is held by a clamp to a ring stand. The glass syringe plunger is pushed in using a microburet that is also clamped to the ring stand. A slow, controlled flow of liquid through the needle can be obtained by leaving a large cushion of air inside the syringe between the glass plunger and the end attached to the tubing. The circular inset shows how the point of the needle should appear after it is clipped.

3. Resuspend the macerated testes in 2 mL of 1X NPB by gently pipeting the solution up and down through a fire-polished, truncated Pasteur pipet with an opening of about 3 mm in diameter.
4. Squirt the sperm suspension through two to four thicknesses of cheesecloth placed into a funnel, and collect the solution into a 15-mL tube (we use round bottom polypropylene tubes; Fisher, cat. #: 14-956-1J). Rinse the forceps and dish with 8 mL of 1X NPB, and force this through the cheesecloth into the 15-mL tube. With a gloved hand, fold the cheesecloth and squeeze any remaining liquid through the funnel into the 15-mL tube.
5. Pellet the sperm by centrifugation at 1500*g* for 10 min at 4°C (we use a Sorvall HB-6 or similar swinging bucket rotor fitted with the appropriate adapters). Resuspend sperm in 8 mL NPB and repellet by centrifugation at 1500*g* for 10 min at 4°C.
6. Resuspend pellet in 1 mL NPB with a cut plastic pipet tip, warm the suspension to room temperature, and add 50 µL of 10 mg/mL lysolecithin. Mix gently and incubate for 5 min at room temperature.

mally, the high-speed spin should begin within 45–60 min of dejellying the eggs. Gently expel eggs manually from each frog into a large dish of 1X MMR, and collect unbroken eggs with even pigmentation. Good eggs can also be collected from the 1X MMR in the frog buckets. Total volume of eggs should be 100 mL or greater before dejellying.

3. Remove as much MMR as possible from the eggs. Dejelly eggs in 2% cysteine in XB salts (no HEPES/sucrose). Add a small amount at a time, swirl eggs, and partially replace with fresh cysteine several times during dejellying. Remove broken eggs with a pipet during dejellying. Dejellying can be performed separately for different batches of eggs, and batches that show breakage or egg activation are discarded.
4. Wash eggs in XB (with HEPES/sucrose). We use about 35 mL for each wash, and do four washes.
5. Wash eggs in CSF-XB with protease inhibitors. We do two 25-mL washes.
6. Using a wide-bore Pasteur pipet, transfer eggs into Beckman ultraclear tubes. For these volumes, we typically use 14 × 95 mm tubes (cat. no.: 344060; Beckman, Fullerton, CA 344057). If multiple tubes will be used, try to transfer an equal volume of eggs per tube. Remove as much CSF-XB as possible, and replace with about 1 mL of Versilube F-50.
7. Spin in a clinical centrifuge at room temperature for about 60 s at 1000 rpm (150*g*) and then 30 s at 2000 rpm (600*g*). Eggs should be packed after this spin, but unbroken. Versilube should replace the CSF-XB between the eggs, and an inverted meniscus between the Versilube and displaced CSF-XB should be clearly visible. Remove the excess CSF-XB and Versilube, and then balance the tubes.
8. Spin the tubes in rubber adapters for 10 min at 16,000*g* at 2°C in Sorvall HB-4 or similar swinging bucket rotor to crush the eggs. The eggs should be separated into three layers: lipid (top), cytoplasm (center), and yolk (bottom). Collect the cytoplasmic layer from each tube with an 18-gage needle by inserting the needle at the base of the cytoplasmic layer and withdrawing slowly. Transfer cytoplasm to a fresh Beckman tube on ice. If large volumes of darkly pigmented eggs are used, the cytoplasmic layer may be grayish rather than golden at this step. After a second spin to clarify this extract, it should be golden.
9. Add protease inhibitors to the isolated cytoplasm (do not add cytochalasin); recentrifuge the cytoplasm in Beckman tubes for an additional 10 min at 16,000*g* to clarify, again using a swinging bucket rotor. Collect the clarified cytoplasm as before. Expect to get obtain 0.75–1 mL cytoplasm/batch of eggs collected from one frog.
10. Add 1/20 vol of the ATP-regenerating system (energy mix). Transfer the clarified cytoplasm into TL100.3 thick-wall polycarbonate tubes (Beckman 349622). Tubes hold about 3 mL each and should be at least half full.
11. Add $CaCl_2$ to each tube to a final concentration of 0.4 m*M*; this inactivates CSF and pushes the extract into interphase. Incubate at room temperature for 15 min and then balance for the high-speed spin.
12. Spin tubes in a Beckman tabletop TL-100 ultracentrifuge in a TL100.3 rotor (gold top; fixed angle) at 70,000 rpm for 1.5 h at 4°C.

7. Mix the reaction by gentle pipeting (using a clipped yellow tip). Incubate for 10 min at room temperature; sperm will now be visibly swelled if diluted into Hoechst as before and observed with a 10X–20X objective.
8. While sperm are swelling in reaction mixture, collect eggs from individual frogs and dejelly them in 2.5% cysteine hydrochloride in 1X MMR (pH 8.0 with NaOH).
9. Under the dissecting microscope, inspect the eggs released from each frog for general health (eggs with even pigmentation and that remain round after dejellying). Draw the healthiest eggs into a wide-bore Pasteur pipet and transfer them to the square space in the injection dish. We generally fill the square space with eggs such that no space is left between the eggs. After about 5 min in 0.4X MMR + 6% Ficoll, the eggs will pierce easily.
10. Dilute the sperm into sperm dilution buffer (SDB) at 1:25–1:100 (such that the final dilution is 1:250–1:1000 or a concentration of 1–2 sperm/10–15 nL injection volume). For some enzymes, such as *Not*I or *Xba*I, add $MgCl_2$ to 5 m*M* to aid enzyme action.

    Before removing sperm from the stock tube or from the dilution used for injection, **always mix thoroughly** with a cut yellow tip, since sperm will rapidly settle out of the suspension.
11. Use a piece of Tygon tubing attached to a yellow tip (as previously described for Sigmacoting needles) to draw up the dilute sperm suspension and back-load the needle. Reattach the needle to the micromanipulator, and turn the air pressure up just slightly so that solution begins to flow from the needle tip (seen under the microscope as a schlearing solution of a different density). Owing to the low air pressure, solution will flow out of the needle only when the tip enters the liquid.
12. Transplant sperm nuclei into unfertilized eggs. The rate of flow should be robust enough that the needle does not reverse flow or clog with cytoplasm during injections and slow enough to be manageable. At the flow and injection rates we generally use, about 10-nL vol is delivered in each injection, so a 1:500–1:1000 dilution of the original sperm stock allows approximately one sperm to be injected in that volume. Move the needle fairly rapidly from egg to egg, piercing the plasma membrane of each egg with a single, sharp motion and then drawing the needle out more slowly. The angle of the needle should be perpendicular to the membrane surface (rather than glancing) to avoid tearing the plasma membrane.

    A hole about the diameter of the needle tip should be visible on the egg and should remain open for about 5 s after injection; when the flow is too low, the hole created in the egg by the needle instantly closes over after injection and little or no volume is delivered. When the flow is too rapid, the surface of the egg near the injection site may ripple or the site of injection may expand in size significantly. If the needle becomes clogged with cytoplasm, bring the tip to the air-liquid interface of the dish. Sometimes the surface tension of the interface removes the cytoplasm plug in the end of the needle. If a needle tip is too narrow, or if it becomes partially clogged with debris during transplantations, the injected nuclei will be damaged during transplantation, and haploid embryos will result.

period (>24 h) before maturation. Transfer defolliculated oocytes to fresh media, and change these media several times to remove traces of yolk and debris.
2. Prepare sperm nuclei as previously described (**Subheading 3.1.1.**)
3. Add 1–5 μm progesterone to oocytes maintained in MBS + 1 mg/mL BSA to begin maturation.
4. Determine when sperm nuclei should be transplanted. A general rule to follow is that oocytes should be ready for fertilization in about 2X the amount of time taken to get from progesterone addition to germinal vesicle breakdown (GVBD; appearance of white spot in the animal hemisphere). We typically add 5 μm progesterone in the evening after defolliculating oocytes (5–7 PM), incubate oocytes at 18°C overnight and during the next day, and inject sperm 20–25 h after progesterone addition (*see* **Note 2**).

   The most common mistake made is not allowing oocytes sufficient time after maturation before injecting the sperm nuclei. Oocytes must be able to respond to pricking by a needle with a vigorous cortical contraction before sperm are transplanted, or no development will occur. Even after oocytes first become responsive to pricking, they are probably not fully competent to support embryonic development immediately and should be incubated an additional 3–4 h at 18°C. Since there is probably quite a bit of variability between batches of oocytes from different frogs and between frogs from different colonies, the optimal timing should be determined by prick-activating a small number of test oocytes at several times during the incubation period to determine when they become responsive.
5. Dilute and transplant sperm nuclei as described in the transgenesis protocol. There is no need to swell the nuclei in interphase extract. We have used slightly lower dilutions of sperm than are used for transgenesis for this protocol (such that two to three sperm may be deposited into some eggs) and have done these injections in 0.4X MMR without Ficoll. Use a 40–60 μm wide needle tip to transplant the sperm as described for transgenesis. When successful, oocytes should pierce very easily for injection, and membrane texture should not seem at all rubbery. There should be a normal cortical contraction in the animal hemisphere after activation, and the injected, matured oocytes (eggs now) should look and later cleave like fertilized eggs. When testing this method, approx 25% of the in vitro matured oocytes developed into blastula-gastrula stages. Of these, the majority developed into tadpoles, and were apparently morphologically normal and raised for months.

4. Notes

4.1. Factors Affecting the Success of Nuclear Transplantation-Based Transgenesis

1. Egg quality is a major factor that contributes to the level of postgastrula development, which is obtained from sperm nuclear transplantation. To obtain good postgastrula development, eggs must be generally healthy. In particular, they should have even pigmentation and should be firm enough to hold their shape well after dejellying. In addition, it is important that they do not become acti-

4.3. Future Prospects in the Frog: Knockouts and Genetics

7. Except for studies where gene function has been inhibited by the expression of dominant negative mutations *(29–31)* or maternal mRNAs where degraded following injection with oligonucleotides *(12–15)*, it has been difficult to inhibit the function of genes in the early embryo specifically. In the future, we also hope to combine transgenesis with antisense *(32–34)* and ribozyme *(35–37)* technologies in order to deplete specific gene products from *Xenopus* embryos.

The advantages of the frog system are numerous, but one major disadvantage is that it has not been exploited at the genetic level. The method for transgenesis we have developed can be adopted for an insertional mutagenesis scheme. Since *Xenopus laevis* is pseudotetraploid and has a long generation time, we suggest using *Xenopus tropicalis*, which is diploid and has a generation time of around 4–6 mo *(38)*. For similar reasons, *Xenopus tropicalis* will also be the species of choice for doing targeted mutations.

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References

1. Kroll, K. L. and Amaya, E. (1996) Transgenic Xenopus embryos from sperm nuclear transplantation reveal FGf signaling requirements during gastrulation. *Development* **122**, 3173–3183.
2. Kroll, K. L. and Gerhart, J. C. (1994) Transgenic *X. laevis* embryos from eggs transplanted with nuclei of transfected cultured cells. *Science* **266**, 650–653.
3. Leno, G. H. and Laskey, R. A. (1991) DNA replication in cell-free extracts from *Xenopus laevis*, in *Methods in Cell Biology*, vol. 36 (Kay, B. K. and Peng, H. B., eds.), Academic, San Diego, CA, pp. 561–579.
4. Murray, A. W. (1991) Cell cycle extracts, in *Methods in Cell Biology*, vol. 36 (Kay, B. K. and Peng, H. B., eds.), Academic, San Diego, CA, pp. 581–605.
5. Newmeyer, D. D. and Wilson, K. L. (1991) Egg extracts for nuclear import and nuclear assembly reactions, in *Methods in Cell Biology*, vol. 36 (Kay, B. K. and Peng, H. B., eds.), Academic, San Diego, CA, pp. 607–634.
6. Schiestl, R. H. and Petes, T. D. (1991) Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **88**, 7585–7589.
7. Kuspa, A. and Loomis, W. F. (1992) Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA. *Proc. Natl. Acad. Sci. USA* **89**, 8803–8807.

23. McGinnis, N., Kuziora, M. A., and McGinnis, W. (1990) Human *Hox-4.2* and *Drosophila Deformed* encode similar regulatory specificities in *Drosophila* embryos and larvae. *Cell* **63,** 969–976.
24. Brakenhoff, R. H., Ruuls, R. C., Jacobs, E. H., Schoenmakers, J. G., and Lubsen, N. H. (1991) Transgenic *Xenopus laevis* tadpoles: a transient *in vivo* model system for the manipulation of lens function and lens development. *Nucleic Acids Res.* **19,** 1279–1284.
25. Dillon, N., Kollias, G., Grosveld, F., and Williams, J. G. (1991) Expression of adult and tadpole specific globin genes from *Xenopus laevis* in transgenic mice. *Nucleic Acids Res.* **19,** 6227–6230.
26. Awgulewitsch, A. and Jacobs, D. (1992) Deformed autoregulatory element from *Drosophila* functions in a conserved manner in transgenic mice. *Nature* **358,** 341–344.
27. Westerfield, M., Wegner, J., Jegalian, B. G., DeRobertis, E.M., and Puschel, A.W. (1992) Specific activation of mammalian Hox promoters in mosaic transgenic zebrafish. *Genes Dev.* **6,** 591–598.
28. Morasso, M. I., Mahon, K. A., and Sargent, T. D. (1995) A *Xenopus* distal-less gene in transgenic mice: conserved regulation in distal limb epidermis and other sites of epithelial-mesenchymal interaction. *Proc. Natl. Acad. Sci. USA* **92,** 3968–3972.
29. Herskowitz, I. (1987) Functional inactivation of genes by dominant negative mutations. *Nature* **329,** 219–222.
30. Christian, J. L., Edelstein, N. G., and Moon, R. T. (1990) Overexpression of wild-type and dominant negative mutant vimentin subunits in developing *Xenopus* embryos. *New Biol.* **2,** 700–711.
31. Amaya, E., Musci, T. J., and Kirschner, M. W. (1991) Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell* **66,** 257–270.
32. Harland, R. and Weintraub, H. (1985) Translation of mRNA injected into *Xenopus* oocytes is specifically inhibited by antisense RNA. *J. Cell Biol.* **101,** 1094–1099.
33. Melton, D. A. (1985) Injected antisense RNAs specifically block messenger RNA translation *in vivo*. *Proc. Natl. Acad. Sci. USA* **82,** 144–148.
34. Nichols, A., Rungger-Brändle, E., Muster, L., and Rungger, D. (1995) Inhibition of *Xhox1A* gene expression in *Xenopus* embryos by antisense RNA produced from an expression vector read by RNA polymerase III. *Mech. Dev.* **52,** 37–49.
35. Cotten, M. and Birnstiel, M. L. (1989) Ribozyme mediated destruction of RNA *in vivo*. *EMBO J.* **8,** 3861–3866.
36. Zhao, J. J. and Pick, L. (1993) Generating loss-of-function phenotypes of the *fushi tarazu* gene with a targeted ribozyme in Drosophila. *Nature* **365,** 448–451.
37. Bouvet, P., Dimitrov, S., and Wolffe, A. P. (1994) Specific regulation of *Xenopus* chromosomal 5S rRNA gene transcription *in vivo* by histone H1. *Genes Dev.* **8,** 1147–1159.
38. Tymowska, J. and Fischberg, M. (1973) Chromosome complements of the genus *Xenopus*. *Chromosoma* **44,** 335–342.